Synopsis

Immunomodulatory protein hydrolysates and their efficacy in Protein Energy Malnutrition

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Chapter 1: Introduction and Review of Literature

Protein energy malnutrition (PEM) continues to be a pervasive nutritional crisis worldwide, especially in developing countries. Growth and developmental impairments are the hallmarks of PEM. PEM ~~also~~ increases the loss of critical storage proteins in the body by anorexia, vomiting, diarrhea, and hypermetabolic losses of cellular constituents, including essential amino acids (Collins, 2003). PEM can affect various organ systems, and its degree of severity depends on several factors, such as the rate of protein turnover, rate of protein synthesis, cell proliferation rate, and role of individual nutrients in metabolic pathways (Marchingo & Cantrell, 2022). Other manifestations of PEM include increased oxidative stress that contributes to immune depression, organ damage, decreased intestinal barrier function, and disruption of the dynamic balance of gut microflora, which in turn may lead to gut dysbiosis and secondary damage (Wang et al., 2020). Alterations in the gut are characterized by changes in the architecture of gut mucosa, such as flattened microvilli cells, reduced lymphocyte counts in Peyer's patches, and decreased immunoglobulin A secretion (Reboldi & Cyster, 2016)

Children with protein energy deficiency are known to have an increased predisposition to the risk of infection-related morbidity and mortality. PEM, immunity, and infection are closely interlinked and form a vicious cycle, and as it continues, it leads to increased mortality in children (Keusch, 2003). Protein deficiency compromises the defense mechanisms and impairs the development of the normal immune system. PEM weakens the immune system and compromises the secretory immunoglobulin A (IgA) antibody response, cell-mediated immunity, the complement system, and the bactericidal function of neutrophils (Mantis et al., 2011). The first line of defense, i.e., the innate immune system, is affected in PEM, and as a result, a higher prevalence of infections is found in PEM. Infections aggravate the previously existing poor nutritional status or trigger malnutrition through disease pathogenesis. As this cycle perpetuates, it ultimately leads to death. Studies have highlighted increased mortality in

children with moderate malnutrition and are not restricted to severe forms (Darling et al., 2020). Protein deficiency is responsible for immunologic deficiency in the humoral and cellular subsystem, predisposing a child with PEM to infections and increased mortality (Gorse et al., 1989).

The gastrointestinal tract (GI tract) plays dual roles in human physiology, primarily one of digestion and uptake of nutrients, while the latter includes a more daunting task of maintaining immune homeostasis (Gu et al., 2021). The unique architecture of the GI tract assists in carrying out both functions; multiple levels of infolding result in an immense overall surface area that allows maximal nutrient absorption while housing the largest number of immune cells in the body (Mason et al., 2008). The intestinal epithelial barrier, along with the gut-associated lymphoid tissue (GALT), houses the largest repertoire of immune cells that plays a fundamental role in determining the immune status of an individual (Childs et al., 2019). The microbial composition and their metabolites play a protective role in different host processes, such as energy harvest, recovery from inflammation and infection, resistance to autoimmunity, and endocrine signaling, which affects brain function through the gut-brain axis (Martin et al., 2018).

Gut homeostasis is maintained due to cross talk between luminal surface organisms and immune cells to ensure tolerance of commensal microorganisms versus appropriate defensive reactions to pathogens (Okumura & Takeda, 2017). Gut dysbiosis is yet another characteristic feature of PEM that affects the absorption of nutrients, their digestion, storage, and their utilization. In the case of severe acute malnourished children, there were alterations in the gut microbiome with an increase in Proteobacteria and a decrease in Bacteroides (Iddrisu et al., 2021). Although inadequate access to sufficient nutrition is the main cause of malnutrition, the gut microbiota has also been implicated in this condition (Lim et al., 1988; Wang et al., 2020). Alterations in the gut microbiota, characterized by an increase in the phylum Proteobacteria and a decrease in Bifidobacterium and Lactobacillus species, are associated with episodes of diarrhea (Martin et al., 2018; Rinninella et al., 2019), and diarrhea is a major causative factor in malnutrition of children, especially those in low-income countries (Kiewiet et al., 2018). Microbial switch with an increase in Bifidobacterium and Lactobacillus species and a corresponding decrease in

Proteobacteria improves intestinal digestive and absorptive functions of nutrients, particularly vitamins, with a consensual stimulation of the GALT, that reduces the risk of contracting allergies (Jablonska et al., 2015).

Milk proteins are at the forefront of global attention due to their significant impact on human nutrition and health. Dairy proteins have maximum biological value and are rapidly absorbed and utilized in the body compared to other protein sources. However, its composition, functional and nutritional properties are highly variable based on the mammalian source, lactation period, genetic, nutritional, and physiological factors, etc. (Pietrzak-Fiecko & Kamelska-Sadowska, 2020). The milk proteins of each mammalian species are quite distinct and may be significant in terms of relative nutritional importance and biofunctional properties (Palacios et al., 2010). Differences exist in the content of different casein proteins (CN), such as αs1-CN, αs2-CN, β-CN, and k-CN, from species to species. For example, β-CN is the significant major protein in both camel and goat milk (Al-Shamsi et al., 2018), whereas αs1-CN and β-CN are the main forms in horse and donkey milk (Vincenzetti et al., 2008). The content of proteins also varies among different dairy proteins; for Eg. camel milk lacks β-lactoglobulin while some immunoglobulins and other minor proteins are unique in some mammalian milk and their colostrum fractions (Shu et al., 2018).

The bioactivity of milk proteins can further be enhanced by enzymatic hydrolysis. The hydrolyzed peptides have the potential to play important roles in preventive and therapeutic healthcare. Protein hydrolysates that exhibit several physiological and biofunctional properties are more easily absorbed in the human gut than intact proteins. Some of these bioactivities include immunomodulatory (LeBlanc et al., 2002), antimicrobial (Meisel & Bockelmann, 1999), antioxidant (Tonolo et al., 2019), and peptidase inhibitory activity, among others. Alcalase is a renowned food-grade endoprotease from *Bacillus licheniforms* that is highly effective in hydrolyzing αs-casein and β-lactoglobulin than other proteases. Alcalase is a broad substrate enzyme that hydrolyses peptide bonds with aromatic amino acid residues that increase methionine and aromatic amino acids (Tacias-Pascacio et al., 2020). Alcalase also generated more potential bioactive peptides than other food-grade proteases, as revealed in the case of

camel milk (Al-Shamsi et al., 2018), goat & cattle milk (Shu et al., 2018), and other dairy proteins (Halavach et al., 2020).

The conventional practice of restoration with high proteins has been effective only to a certain extent and has not been able to restore the immune system completely. This is further justified by studies that stated that nutrient repletion towards malnutrition has seldom been able to restore full-fledged immune competence (UNICEF, 2019). There is now a need for alternate approaches concerning nutrient supplementation that needs to be addressed in the diet of PEM patients for better immune responses.

Statement of problem:

The conventional practice of alleviating PEM through restoring high-protein diets has been met with limited success. While numerous studies have been reported on bioactive peptides, protein fractions, and dairy products from bovine milk, comparative studies across different dairy sources and their applications in alleviating PEM have not received sufficient attention from scientific research. Moreover, dairy protein hydrolysates exhibit enhanced immunomodulatory properties, which can play a vital role in restoring the depleted immune system in PEM individuals. Therefore, the current study was undertaken to address this gap in screening, comparing, and evaluating the efficacy of various dairy protein hydrolysates for their antioxidant, antimicrobial, and immunological significance as potential dietary interventions for PEM.

Furthermore, dietary interventions for alleviating PEM have not considered the role of dairy protein hydrolysates in influencing gut immunity and the critical role of the gut microbiome in the pathogenesis of PEM, and the microbial shifts occurring due to dietary replenishments.

The paucity of such information is a critical unmet need because such investigation not only has the potential to contribute to nutrition but also to the discretion of incorporating milk protein hydrolysates for the formulation of functional/ therapeutic foods that can be used to combat PEM in children

Objectives:

1. **Preparation of protein hydrolysates from milk proteins with enhanced immune-modulatory effect and determining its efficacy in *in-vitro* models:**
   Dairy proteins from different mammalian sources and respective hydrolysates were compared and assessed for their antioxidant and immunological significance. The results are discussed in Subchapter 2.3.

2. **Validation of the immune-modulatory protein hydrolysates in Protein Energy Malnutrition (PEM) induced animals models through gene expression studies:**
   The chapter is subdivided into three subchapters, where large-scale production of milk protein hydrolysates is discussed in subchapter 3.3.1. The efficacy of milk protein hydrolysates was validated in PEM induced rats, and the biochemical findings and antioxidant activities are addressed in Subchapter 3.3.2. Investigations involving the expression of immunoregulatory genes and whole genome sequencing of fecal samples were undertaken to understand the impact of protein deficiency and protein replenishments on modulating gut dysbiosis, and their microbiota is assessed in subchapter 3.3.3.

3. **Formulation of beverage mix incorporating the immuno-modulatory protein hydrolysates for alleviation of PEM in children:**
   The chapter is subdivided into three subchapters, where subchapter 4.3.1 discusses the incorporation of milk protein hydrolysate in the beverage mix, their organoleptic properties, optimization, and characterization. Animal studies involving Wistar rats were done to determine the efficacy of these protein hydrolysate-incorporated beverage mixes against a standard control beverage and were evaluated in section 4.3.2. Investigations of the effect of these mixes on PEM-induced gut dysbiosis and their microbiota are discussed in section 4.3.3.

Chapter 2

2.1.Materials

Milk from cows, buffalo, and donkeys was collected from dairy farms in Nanjangud Road and Yadavgiri (Mysore, India). Milk samples were stored at -80 ºC and were later freeze-dried in a freeze dryer (Alpha 2–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Freeze-dried goat milk and camel milk were procured from Caltest Laboratories Pvt . Ltd. (Gujarat, India), Bovine whey protein concentrates were procured from Mahan Protein Pvt. Ltd. (Delhi, India), Bovine colostrums from Biostrum Nutritech Pvt. Ltd. (Gujarat, India), while casein was brought from Casein India Pvt. Ltd. (Mumbai, India)

2.2.Methodology for Preparation of milk hydrolysates, its characterization, and studying bioactivity

The proximate composition of the milk powders was estimated (AOAC, 2000). Milk powders were defatted using food-grade hexane. Hydrolysis of defatted milk powders was carried out using food-grade alcalase. The aliquots of hydrolysates were withdrawn at two different timelines, one at 10 min intervals (i.e., 0–30 min) and the other at 1 h intervals from 1 to 4 h. The degree of hydrolysis (DH) was determined by the TCA method adopted by Hoyle and Merritt (1944). The hydrolysates obtained at the end of 4 h were freeze-dried for further analysis. Skimmed milk protein hydrolysates (MPHs) were characterized by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Pardo, 2002). Milk proteins and respective hydrolysates were analyzed by reverse-phase high-performance liquid chromatography (RP- HPLC) following the method described by Vincenzetti et al. (2008). The amino acids were estimated in an amino acid analyzer by Simpson et al. (1976). DPPH, ABTS, and FRAP antioxidant activities of the native and hydrolyzed milk proteins were determined. The immunomodulatory activity of these hydrolysates was checked *in vitro* through RAW 264.7 mouse macrophage cell line. The phagocytic ability of macrophages was measured by a neutral red uptake assay (Repetto et al., 2008). The assay involves the measurement of Nitric oxide (NO) by determining the nitrite levels in the medium (Wu & Yotnda, 2011a, 2011b)

2.3. Results and discussion

Screening of antioxidant and immunomodulatory properties of skimmed milk protein hydrolysates

Hydrolysis of all the milk samples, i.e., bovine skimmed milk (BSM), bovine WPC, bovine casein (BCa), bovine skimmed colostrum (BCo), buffalo skimmed milk (BM), camel skimmed milk (CM), donkey skimmed milk (DM), and goat skimmed milk (GM) were carried out using food-grade alcalase for 4 h. It was found that all hydrolysates showed the highest DH, but they reached saturation after 2 h, beyond which no significant increase was found. The results were similar to those observed previously (Jung et al., 2016), which reported that alcalase treatment over 30 min yielded no significant differences in the non-protein nitrogen under optimal conditions. Bovine colostrum had a lower hydrolytic efficiency than other milk proteins, which could be attributed to a higher proportion of casein fraction, which is resistant to hydrolysis by alcalase. The protein profile of native milk from cattle and non-cattle milk was compared with its hydrolysates along with the standard molecular marker and a milk marker as reference using tricine-SDS PAGE. Lactoferrin, bovine serum albumin (BSA), caseins (β, and κ), β-Lactoglobulin (β-LG), α-Lactalbumin (α-La) were detected in BSM. Upon hydrolysis, these protein bands disappeared except for casein proteins which were visible as a faint band. Tricine SDS profile of non-cattle milk samples exhibited marked heterogeneity showing distinctive electrophoretic patterns compared to cattle milk. RP-HPLC was carried out to ascertain further the presence of low molecular weight peptides after hydrolysis by alcalase. The chromatograms of native milk proteins revealed prominent peaks between 30-60 min. The chromatogram of hydrolyzed proteins exhibited several small peaks of eluting below 20 min, marked by the corresponding disappearance of major peaks eluted in between 30-60 min. The increase in the number of these smaller peaks shows the presence of several lower molecular weight peptides, thus confirming hydrolysis by alcalase and supporting the observations of Tricine SDS-PAGE and DH. Higher concentrations of hydrophobic amino acids and sulfur-containing amino acids were found in hydrolysates of whey, casein colostrum, buffalo milk, and camel milk than in the native milk.

In the case of the DPPH assay, the antioxidant activity of hydrolysates was better, as observed in camel milk hydrolysate (0.88 mg mL^{-1}) and whey protein hydrolysate (1.16 mg mL^{-1}), while the least was in donkey milk hydrolysate (5.37 mg mL^{-1}). Milk protein hydrolysates were also more effective in quenching the ABTS free radical than native milk protein, the IC_{50} value of whey protein hydrolysate (WPH) was the least among at 54.26 and by far the most effective among all milk hydrolysates. The higher free radical scavenging activity in these hydrolysates is probably due to the nature of peptides or amino acids that can donate protons to electron-deficient radicals leading to the generation of stable products (Shahidi & Zhong, 2010). FRAP activity revealed that WPH (25.00 ± 0.16 mM) and bovine casein hydrolysate (25.14 ± 0.12 mM) were comparatively better in reducing more ferrous to ferric form. Compared with native milk, the hydrolysates were more capable of reducing ferrous to ferric form, except for BSM and DM. The difference in the antioxidant activity of protein hydrolysates might be attributed to the specific peptide or the composition of amino acids (Everett et al., 1995). The cytotoxic effect of MPH was evaluated using the RAW 264.7 cells through WST assay. The WST assay indicated that most of the protein hydrolysates did not show cytotoxic effects on RAW 264.7 cells at concentrations ranging from 0 to 1000 µg mL^{-1} of equivalent protein except for bovine casein hydrolysate which revealed decreased cell viability at 1000 µg mL^{-1} . Thereafter, the cells were treated with the hydrolysates in 500 μg/mL concentration during subsequent experiments. Protein hydrolysates of skimmed whey, buffalo, camel, and goat milk enhanced the phagocytic activity of RAW 264.7 cells. Among them, WPH and BMH exhibited the highest promoting activity, wherein macrophage phagocytosis was increased by 44.7 % and 43.8 %, respectively ($P<0.05$). Protein hydrolysates of buffalo and whey significantly elevated levels of NO in comparison to the native milk. The NO concentration observed in WPH was 8.07 ± 0.40 µM while it was 6.86 ± 0.64 µM in the case of buffalo milk protein hydrolysate (BMH). BMH and WPH showed good antioxidant activity and phagocytosis ability and hence were chosen for further validation of immune modulation in protein deficiency-induced rats

Chapter 3.

3.1. Materials

Whey protein concentrates were procured from Estrella life sciences, while fresh buffalo milk was collected from the dairy farm in Nanjangud Road (Mysuru, India). Food-grade alcalase was purchased from Novo Nordisk Co. (Novo Nordisk A/S, Bagsværd, Denmark). Serum protein

and other biochemical assays were done using the Agappe kit (AGAPPE Diagnostics, Switzerland), sIgA ELISA kit was procured from Krishgen (India). Primers were procured from Takara Bio, while the rest of the kits involved in gene expression studies were purchased from Thermo Scientific (USA). All other chemicals and solvents used throughout the investigation were of analytical grade. Triple distilled water was used for the entire analysis.

3.2.Methodology

3.2.1. Pilot-scale production of these two milk proteins was carried out. The buffalo milk samples were pooled, defatted by cold centrifugation, spray-dried, and stored at -20 ºC. WPC / Buffalo milk was mixed with water in a ratio of 1:15 (w/v) in a large bioreactor to obtain a uniform slurry. Hydrolysis was continued for 8h by maintaining the pH at 8. Afterward, the hydrolysates were centrifuged in a tubular centrifuge, and the supernatant was collected and spray-dried to obtain milk protein hydrolysates. The protein content of milk protein hydrolysates was estimated, and their yield was calculated.

3.2.2. Weanling male rats of the Wistar strain (22 - 24 g body weight) were separated from their mothers and fed a standard AIN93-G diet and water ad libitum for 2 days. PEM was induced by feeding the rats with 5% protein, i.e., Moderate PEM group (M.PEM), and 1% protein, i.e., Severe PEM group (S.PEM) based on the method of Mgbodile and Campbell (1972). Phase I involved the control series receiving a standard AIN-93G diet containing 15% protein, and the undernourished series were fed a hypoproteic diet. Casein was replaced by starch in the 5% and 1% protein diet to make it isocaloric, and diets were provided for 3 weeks. Phase II commenced after three weeks, and the rats were subdivided into seven groups, i.e., Moderate PEM (M.PEM) was subdivided into three groups based on the source of protein replenishment. The first group was replenished with 15% casein (MP-RC), the second group with 7.5% BMH and 7.5% casein (MP-RBC), and the last group received 7.5% WPH and 7.5% casein (MP-RWC). Severe PEM (S.PEM) too was categorized likewise into three groups and were replenished with 15% casein (SP-RC), 15% BMH: 15% casein (SP-RBC), and 7.5% WPH: 7.5% casein (SP-RWC) while casein at 15% was continued, during replenishment for the control group (C-RC). Nitrite in samples was used to indicate

nitric oxide (NO) production. The stable product from NO and molecular oxygen can be estimated according to the Griess reagent method (Tsikas, 2007). Sodium nitrite (0–100 μM) was used as the reference standard, and absorbance was measured at 540 nm. The protein carbonyls were evaluated by the 2,4-dinitrophenylhydrazine (DNPH) alkaline method, and Lipid peroxide can be indirectly measured from malondialdehyde (MDA) levels. The stress markers of lipid peroxide in the ileum homogenate were analyzed by the thiobarbituric acid reaction (TBA) method (Mesquita et al., 2014).

3.2.3. Ileum samples were homogenized in PBS containing butylated hydroxytoluene. They were later stored at −80 °C, freeze-thawed, and RNA was extracted as described by Walther et al. (2008). Gene expression studies were carried out using primers of selective cytokines involved in sIgA production, while IgA quantification was carried out through an ELISA kit. Stool samples were collected at two different intervals. Fecal samples were collected, and whole genome sequencing was performed on all groups using long-read sequencing. Raw sequencing reads were base-called and assembled for the whole genome-based taxonomic analysis, as mentioned in Kumar *et al*. (2022).

3.3.Results and Discussion

3.3.1. Pilot-level processing of whey protein hydrolysates and buffalo milk hydrolysates

The yield of protein hydrolysates for whey protein hydrolysate was 28%, and buffalo milk protein hydrolysates were 25%.

3.3.2. Evaluation of biochemical, antioxidant, and hematological parameters in whey and buffalo milk protein hydrolysate supplemented PEM rats

The weaning period is associated with a high growth rate; in the normal rats, the body weight increased by 71.7 ± 5.1 g in three weeks, while the M.PEM rats gained only 2.4 ± 0.91 g, and the S. PEM group lost 4.7 ± 1.4 g in the same period. This accounts for 86.7% of the weight loss in M.PEM and >100% of the weight loss in S.PEM rats compared to that of control rats, which is indicative of growth impairment. This also marked the successful establishment of M.PEM and S.PEM. The findings were similar to that of Collins et al. (2013), wherein PEM induction showed retarded animal growth. The gain in body weight accelerated rapidly upon

protein replenishment in both S.PEM and M.PEM groups. On induction of PEM, there was a decrease in the hematological parameters; hemoglobin had reduced in moderate and severe PEM ($P<0.0001$), and a linearity was observed in the hemoglobin content concerning the severity of PEM. RBC count indicative of red blood cell volume also reduced on PEM induction in proportion to the degree of malnutrition. The fact that hemoglobin is a protein in nature suggests the essentiality of proteins for the formation of red blood cells and hemoglobin. Upon replenishment, casein-fed SP-RC rats did not show improvement in HGB and hematocrit values. At the same time, hydrolysates effectively normalized hematocrit values on par with control in S.PEM replenished rats. WBC count is an indicator of the body's immune status; it was very much lower in protein deficiency-induced rats. The extent of reduction was found to follow the degree of protein deficiency, and it was reduced by more than 50% in M.PEM and 72% in S.PEM rats. The decrease in WBCs contributes to immunosuppression. On replenishment, there was an increase in the WBC count, but it varied by the protein source and the severity of the deficiency ($P<0.01$).

Serum total protein and albumin levels in rats fed with a protein-deficient diet showed a downward trend during the induction phase, which marked the successful establishment of PEM. The complete serum protein was decreased in S.PEM (1.53 ± 0.48 g dL^{-1} ($p < 0.05$) and M.PEM (1.82 ± 0.38 g dL^{-1}) rats compared to that of control rats (2.86 ± 0.50 g dL^{-1}) and was reversed in case of MP-RWC rats. The SGOT and SGPT levels of M.PEM and S.PEM had increased ~ twofold and ~ threefold. An increase in the concentration of SGOT and SGPT levels is related to hepatopathy and hepatic damage. (Torruellas et al., 2014). Replenishment with WPH and BMH hydrolysates was more successful in reducing the SGPT levels. Histopathological results showed that the kidneys of PEM-induced rats were affected with inflammation and degeneration, and replenishment with whey protein hydrolysate successfully reverses this damage. PEM induced rats also had damage in primary and secondary lymphoid organs, i.e., thymus and spleen. Mild depletion of lymphocytes and atrophy of lymphatic follicles was observed in both M.PEM and S.PEM induced rats. Replenishment of WPH and BMH and not casein were successful to an extent in reversing these damages. Oxidative stress markers in serum were elevated in PEM induced rats. The carbonyl content in S.PEM rats was greatly increased two-fold. Upon replenishment, it was observed that there was a decrease in protein carbonyl values except for SP-RC rats (positive control group) which meant mere casein

replenishment wasn't sufficient to negate oxidative stress. Antioxidant markers such as catalase and superoxide dismutase significantly decreased in PEM induced rats. Upon replenishment, all the groups showed increased catalase activity as compared to the control which may have been elevated as a defense mechanism to counter the damage done by PEM induction.

3.3.4. effect of buffalo and whey milk protein hydrolysates in PEM induced gut dysbiosis

The oxidative stress markers were evaluated in the ileum of the small intestine. Nitric oxide (NO) levels in the PEM-induced rats decreased significantly ($p > 0.05$), while protein replenishment proved effective, and the levels became similar to those of the control rats. The lipid peroxide levels in the M.PEM and S.PEM groups ($p < 0.05$) increased by 33% and 41% compared to the control group. The lipid peroxide levels in the M.PEM and S.PEM groups ($P < 0.05$) increased by 33% and 41% compared to those of the control group. Replenishment with whey hydrolysates lowered the lipid peroxide significantly to 0.22 ± 0.04 nM MDA per mg in the SP-RBC group as opposed to the 1.13 ± 0.18 nM MDA per mg in S.PEM-induced rats. The observations in PEM induced rats are similar to previous findings wherein a low protein diet increased lipid peroxidation (Balakrishnan & Anuradha, 1998). Subsequently, the immunomodulatory effect in the gut was evaluated by studying the expression of cytokines such as IL-4, IL-6, IL-10, TGF-1β, and IFN-γ. Pro-inflammatory genes such as IFN-γ and IL-6 were upregulated in S.PEM-induced rats by about 2.17 ± 0.39 and 2.4 ± 0.36 fold. The anti-inflammatory and immune-regulatory cytokines, such as IL-4, IL-10, and TGF-1β, were downregulated in the ranges of 0.05 ± 0.04, 0.3 ± 0.10, and 0.02 ± 0.02 fold, respectively, in S.PEM rats. Upon replenishment, casein and milk hydrolysates seemed to regulate cytokine expression differentially. WPH downregulated the expressions of IL-4, IL-6, and IFN-γ in SP-RWC rats by about 0.37 ± 0.18, 0.17 ± 0.06, and 0.12 ± 0.10 fold, respectively. Gene expression of IL-10 and TGF-1β matched those of the control. sIgA content decreased significantly with PEM induction compared to that of the control. The gut is a complex ecosystem with around 100 trillion microorganisms, predominantly bacteria. The intestinal microbiota and its colonization are crucial factors in maintaining intestinal functions. In PEM-induced rats, Firmicutes accounted for 47.73 ± 7.6% of the total abundance of gut bacteria. At the same time, Bacteroidetes were 28.17 ± 3.2% in the control group. In the S.PEM group, Firmicutes decreased to 28.83±3.8% ($p < 0.01$), and Bacteroidetes increased to 31.03±5.2% of the total abundance of gut bacteria. Protein deficiency induced a significant increase in Proteobacteria

(8.14%), Actinobacteria (2.85%), and Ascomycota (2.89%) in the S.PEM group. Similar findings about the decrease in beneficial bacteria and increase in pathogens were reported (Shin et al., 2015). Primarily, at the phylum level, we observed a beneficial shift towards a higher abundance of Firmicutes and a lower abundance of Proteobacteria post replenishments. Proteobacteria comprises pathogenic species such as Escherichia, Salmonella, Campylobacter, etc. The key species, L. *johnsonii* and L. *reuteri,* increased post replenishment. A significant increase in the relative abundance of L. *johnsonii* post replenishment with casein and milk protein hydrolysates was observed in M.PEM rats. In contrast, replenishment with casein and BMH increased the relative abundance of L. *johnsonii*, and WPH favored L. *reuteri* post replenishment in S.PEM-induced rats.

Chapter 4

4.1. Materials

Mangoes (*Mangifera indica)* were purchased from local markets in Mysuru, India. Other groceries involved in preparing the beverage mix were purchased from Loyal World supermarket (Mysuru, India). The rest of the chemicals and kits used were similar to those that are discussed in Subchapter 3.1.

4.2. Methodology

4.2.1. The basic beverage mix was prepared by mixing mango pulp (45%), whole milk (50%), vegetable fat (2.5%), maltodextrin (3%), bicarbonate (0.5%), pectin (0.5%), and liquid glucose (1.25%) and spray dried (Sinha et al., 2007). 10 g of sugar was added to 90 g of the mix, and the beverage mix was stored in an airtight container until further use. The beverage mix was reconstituted at a 20% level. The basic beverage mix was substituted with whey protein hydrolysate (WPH) and skimmed buffalo milk protein hydrolysate (BMH) at 10–20% to determine the acceptable level through five trained panel members.

4.2.2. The efficacy of the beverage mix was tested in male Wistar rats (22–24 g body weight), and the methodology was similar to Section 3.2.2 with few modifications. The phase I PEM induction period in rats was extended by 4 weeks, wherein PEM

was induced by feeding the rats with 5% protein, i.e., Moderate PEM group (M.PEM), 1% protein, i.e., Severe PEM group (S.PEM)The phase II replenishment was carried out with beverage mixes for 4-11 weeks. The control group receiving an AIN-93 diet was fed an AIN-93 diet with 15% casein (C-RCB). Moderate PEM rats (M.PEM) were subdivided into three groups, the first group was replenished with control beverage mix (MP-RCB), the second group with 10% BMH substituted beverage mix (MP-RBMHB), and the last group received 10% WPH substituted beverage mix (MP-RWPHB). Severe PEM (S.PEM) too was categorized likewise into three groups and was replenished with 15% casein (SP-RCB), 10% BMH beverage mix (SP-RBMHB), and 10% WPH beverage mix (SP- RWPHB). At the end of the depletion and repletion period, the rats were sacrificed through carbon dioxide asphyxiation, and blood was collected by cardiac puncture into heparinized vials.

4.2.3. Gut dysbiosis, microbiome studies, and the effect of replenishment were similar to those discussed in Subchapter 3.2.3.

4.3. Results and Discussion

4.3.1. Formulation of beverage, its characterization, and sensory analysis.

The beverage was formulated with 5%, 10%, and 15% whey and buffalo milk protein hydrolysates. There was a decrease in the color of the mixes with WPH and BMH. The taste, flavor, mouthfeel, and overall acceptability of the basic beverage and the one with 10% incorporation of BMH and WPH showed almost similar scores to that of the control ($P > 0.05$). *In vitro,* protein digestibilities of the control beverage mix (40.75%) were comparatively lesser than whey hydrolysate beverage mix (89.9%) and buffalo mix hydrolysate beverage mix (64%). The increase in protein digestibility can be attributed to the hydrolyzed peptides and the amino acid content in the beverage mix with hydrolysates. There was a significant decrease in the predicted glycemic index values of WPH-beverage mix (66.98 ± 1.4) and BMH beverage mix (66.35 ± 1.5) as against the control beverage mix (71.90 ± 2.1). Differences in amino acid content have been attributed to differences in antioxidative capacities and immunomodulatory activity (Sinha et al., 2007). The sulphur containing amino acids contributing to antioxidant activity was higher in milk protein hydrolysate beverage mixes.

4.3.2. effect of dietary replenishment of beverage mixes on biophysical and pathophysiology in PEM induced rats

We found that while the diet consumption increased for control with age, it decreased for both severe PEM and moderate PEM induced groups, contributing to weight loss, which is the hallmark of protein energy malnutrition. The control rats weighed ~157 g, while body weight decreased in the S.PEM group by 406.5% and in the M.PEM group by 271.6%. Upon replenishment with beverage mixes, we found that diet consumption in PEM induced rats improved drastically and was more or less on par with control rats. The SP-CB rats were not effective in gaining body weight as compared to milk hydrolysate beverage replenished SP-BMHB & SP-WPHB rats. Blood parameters revealed that RBC and WBC decreased drastically in S.PEM induced rats. On repletion, there was an improvement in the hematological parameters. Buffalo milk hydrolysates beverage mixes seemed more effective in reversing the depleted hemoglobin level in SP-RBMHB and MP-RBMH rats. There was a significant decrease in serum total protein and serum albumin levels with a corresponding increase in the severity of PEM.

In contrast, SGOT and SGPT levels increased upon PEM induction, indicating hepatic damage (Batool et al., 2014). Replenishment with all three beverage mixes restored total protein, albumin, and SGPT to that of control. In contrast, only whey hydrolysate beverage was effective in normalizing SGOT levels in MP-RWPHB rats. Histopathology revealed that lymphoid organs, namely the thymus and spleen, and liver and kidney were the most affected and showed functional and morphological alterations upon PEM induction. The control beverage could not reverse thymic follicular atrophy in SP-RC rats. In contrast, buffalo and whey hydrolysate beverage mix effectively reversed the damage in SP-RBMHB and SP-RWPHB rats. There was a significant decrease in the antioxidant activity and an increase in oxidative radicals such as LPO and protein carbonyl in the serum of PEM induced groups. Upon replenishment, all the beverage mixes successfully restored the protein carbonyl levels to that of control while only milk hydrolysate incorporated beverage mixes were effective in normalizing the LPO levels of SP-RBMHB and SP-RWPHB rats while the control beverage mix was ineffective, as observed in SP-RCB rats.

4.3.3. effect of buffalo and whey protein hydrolysates incorporated beverage mixes in PEM induced gut dysbiosis

Animals induced with PEM revealed deleterious effects such as ulcers, submucosal inflammation, and infiltration of inflammatory cells in the ileum**.** Upon replenishment, all three beverage mixes could reverse the lymphoid tissue hyperplasia and inflammation in M.PEM induced rats. The degree and severity of infiltration of inflammatory cells lessened to a certain extent upon replenishment with SP-RWPHB rats. Oxidative stress markers in ileum reveal that the protein carbonyl content in S.PEM induced rats (8.35±1.26 nM/mg ileum) and M.PEM induced rats (7.13±0.48 nM/mg ileum) were significantly elevated. However, beverage replenishment decreased carbonyl content in PEM induced rats, with whey hydrolysate incorporated beverage being the most effective in MP-RWHB rats at 4.07±0.59 nM/mg ileum as against 5.86±0.70 nM/mg ileum in C-RCB rats. Subsequently, the immunomodulatory effect in the gut was evaluated by studying the expression of cytokines such as IL-4, IL-6, IL-10, TGF-1β, and IFN-γ. IFN-γ and IL-6 were upregulated in M.PEM and S.PEM induced rats. The genes responsible for other cytokines, such as IL-4, IL-10, and TGF-1β, were downregulated in PEM induced rats. The three beverage mixes, upon replenishment, seemed to regulate the cytokine regulation differentially. WPH beverage mix downregulated the expressions of IFN-√, TGF-β1, and IL-6 in SP-RWPHB rats by about 0.14 ± 0.01, 0.47 ± 0.07, and 0.39 ± 0.07 folds while in IL-4 and IL-10, it was not significant and matched expression levels to that of control. Beverage mix with BMH downregulated IFN-√ and IL-6 by about 0.01 ± 0.0 and 0.33 ± 0.07 in SP-RBMHB rats, while there was no significant difference in the expression of IL-4 and IL-10 to that of C-RCB rats. Beverage mixes with hydrolysates seemed to regulate the expression of these immunomodulatory cytokines effectively compared to the control beverage mix. sIgA concentration in PEM-induced rats significantly decreased as compared to the control. These results demonstrate that protein deficiency induction during early life decreases expression of the IgA gene, thereby affecting the development of the rat's IgA defense system, which may affect the balance between pathogens and probiotics in the gut (Chandra., 1986). The effect was reversed on replenishment with beverage mixes.

Gut microbiome analysis revealed that at the phylum level, Firmicutes and Bacteroidetes were the two predominant phyla. In PEM-induced rats, Firmicutes accounted for 70.91 ± 5.6% of the

total abundance of gut bacteria. At the same time, Bacteroidetes was 19.47% ± 2.1 % in the control group. In the S.PEM group, Firmicutes decreased to 39.12 ± 3.2% ($p < 0.01$), and Bacteroidetes increased to 20.97% ± 3.6% of the total abundance of gut bacteria. Proteobacteria are often a microbial signature of diseases as many common pathogenic genera such as Brucella, Escherichia, Salmonella, and Rickettsia are found in this phylum (Kumar et al., 2022). The relative abundance of Proteobacteria increased in M.PEM rats (26.39%) and S.PEM (27.58%) as against control rats which had just 2.77%. Upon replenishment with beverage mixes, the two key species, *Lb. johnsonii* and *Lb. reuteri* increased post-replenishment, as seen while pathogens, especially *E.coli*, decreased significantly. The most significant and beneficial change in the microbiome was observed with whey hydrolysate incorporated beverage mixes as compared to others wherein the significant increase in the abundance of probiotic species, that includes *Limosilactobacillus reuteri* (9.63% in SP-RWPHB, 13.15% in MP-RWPHB as against 0.52% in C-RCB rats) *Lb. johnsonii* (11.68% in SP-RWPHB, 13.29% in MP-RWPHB as against 0.43% in C-RCB rats) and *Lactobacillus acidophilus* (1.30% in SP-RWPHB, 1.82% in MP-RWPHB as against 0.07% in C-RCB rats) was observed.

Conclusion

The burgeoning effects of protein deficiency in early life nutrition have pointed towards microbiota and gut dysbiosis causing immune deficiency, further increasing disease susceptibility and mortality. Our investigation aimed to compare native and hydrolyzed proteins of cattle milk and other mammalian milk sources for their bioactivity, especially their antioxidative and immunomodulatory properties. Hydrolysates of skimmed milk proteins of colostrum, buffalo, camel whey, and casein showed good free-radical quenching abilities. Whey and buffalo milk protein hydrolysates seemed to act as a potential source of antioxidants and enhancers for phagocytic activity in RAW 264.7 mouse macrophage cell lines. This suggests that particular amino acids and peptides are involved in discrete molecular mechanisms for antioxidant and nitric oxide production. Whey and buffalo milk protein hydrolysates exhibited better free radical quenching and immunomodulatory properties than the rest.

The stability of these hydrolysates to gastrointestinal digestion and studying their immunomodulatory activity *in vivo* was further carried out in Wistar rats. Our investigation

reveals that protein deficiency exacerbates oxidative stress, disrupts gut function, augments pathogenesis, and decreases the production of sIgA. Replenishment with buffalo and whey protein hydrolysates modulates the immunoregulatory cytokines. It alters the gut microbiota by differentially reducing the relative abundance of pathogens and increasing the abundance of keystone species, particularly L. *johnsonii* and L. *reuteri*. The increase in keystone species may be involved in reversing the hyperplasia and crypt damage caused due to protein deficiency. The preliminary result gives insights into how targeted dietary interventions, particularly in the case of PEM, may prove to be an ideal therapeutic option in preventative healthcare. Buffalo milk protein hydrolysate and whey protein hydrolysate substituted beverage mixes at 10% substitution showed good organoleptic properties and can be recommended for children to improve their immune systems

Publication

1. **Pavan Kumar Prakash** and Jyothi Lakshmi A (2022). Effect of milk protein hydrolysates supplementation on protein energy malnutrition induced gut dysbiosis. Food and Function - **Impact factor - 6.317** **https://doi.org/10.1039/D2FO00714B**
2. **Pavan Kumar Prakash**, Sachin M Eligar, Prakruthi M and Jyothi Lakshmi A (2022). Comparative assessment of antioxidant properties and immunomodulatory properties of various milk proteins hydrolysates and their incorporation in beverage mix**.** Journal of the Science Food and Agriculture. **Impact factor – 4.125 DOI: 10.1002/jsfa.12007**

Under Preparation

- Evaluation of buffalo milk and whey protein hydrolysates in protein energy malnutrition induced rats (Under Preparation)

Abstract in the conference

1. **Conference :** International Conference on feed and nutrition at Singapore

   Title : Evaluation of milk protein hydrolysates in protein energy malnutrition induced rats: Deciphering its state of immunity and antioxidant activity

   Type : Oral Presentation

   Awarded : Best Oral Presentation

2. **Conference :** 28th ICFoST**,**

   Title : Crosstalk between IgA and gut microbiota in protein energy malnutrition induced rats influences antioxidant activity and its state of immunity

   Type : Poster

   Awarded : Best Poster

3. **Conference :** AMIFOST,

   Title : Supplementation Of Milk Protein Hydrolysates in Protein Energy Malnutrition Induced Rats Deciphers a Complex Crosstalk in Alleviating Gut Dysbiosis and State Of Immunity: Their Incorporation And Development Of A Protein Beverage Mix

   Type : Poster

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Candidate signature **Guide Signature**